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HILL TOP RESEARCH, INC.

APPENDIX III

COPY OF PROTOCOL

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PROTOCOL FOR

MODIFIED AOAC GERMICIDAL AND DETERGENT SANITIZING ACTION OF DISINFECTANTS – Rough Plastic
One Step Cleaner Sanitizer

For: Kimberly-Clark Corporation

HTR Ref.: 02-120920-106

December 16, 2002

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1.0 INTRODUCTION

Single-use wipes containing chemical sanitizers suitable for use on lightly soiled, nonporous, food contact surfaces are generally tested by a time kill method where the cidal effect of a specific concentration of chemical agent is measured against both a Gram negative and a Gram positive bacterium over a specified time period. The percent reduction in numbers of test bacteria containing a soil load is calculated as compared to a positive control. Standard practices for testing use the AOAC Germicidal and Detergent Sanitizing Action of Disinfectants Method as described in Chapter 6, Disinfectants, Official Methods of Analysis for AOAC International, 17th Edition, 2000, Section 6.3.03. This method will be modified to test a single-use wipe on a moderately soiled, rough plastic surface in accordance with the EPA Interim Guidance "Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes (April 12, 2001)".

2.0 PURPOSE

To determine the sanitizing action of a wipe containing a chemical agent that can be permitted for use in sanitizing moderately soiled, rough plastic surfaces.

3.0 STUDY SPONSOR AND SPONSOR REPRESENTATIVE

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4.0 TEST FACILITY AND INVESTIGATIVE PERSONNEL

Hill Top Research, Inc.
Main and Mill Streets
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4.0 TEST FACILITY AND INVESTIGATIVE PERSONNEL CON'T.

Telephone No: (513) 831-3114
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5.0 APPLICABLE REGULATION

Federal Insecticide, Fungicide and Rodenticide Act (40 CFR Part 158)
EPA Interim Guidance "Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes (April 12, 2001)"

6.0 RESEARCH STANDARDS

This study will be run according to Good Laboratory Practice Standards (40 CFR Part 160). In-Life Phase and Final Report audits will be conducted by the Quality Assurance Unit of Hill Top Research, Inc.

7.0 EXPERIMENTAL DESIGN

Plastic pans identified as "Nalgene® Polypropylene Pan" [pre-sterilized] are inoculated with a specific number of the test bacteria, containing 5% Fetal Bovine Serum to represent a moderate soil load. Plastic pans were selected as they allow the test surface to be thoroughly submerged in the recovery/neutralization media thus achieving reproducible recovery and immediate neutralization. A deeply grooved pattern was mechanically pressed into the bottom of the plastic pans to simulate a rough plastic surface. The inoculum is placed on the rough area (5 and 7/8" X 8") on the bottom of the pan. A sufficient number of pans are inoculated to represent the specified wiped surface area for testing (wiped surface area per pan measures approximately 13.25" X 11" or 1 square foot). The pans are then wiped with a wipe (12" X 12") containing the chemical germicide for a specified period of time. At predetermined exposure time(s), 30 seconds, the remaining chemical agent on the pan is inactivated, and the surviving bacteria are enumerated. The percent reduction in numbers of test bacteria is then calculated. The percent reduction in numbers of bacteria is calculated from a positive control.

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8.0 PROPOSED EXPERIMENTAL STARTING AND EXPERIMENTAL TERMINATION DATES

Proposed Experimental Starting Date:	November 12, 2002
Proposed Experimental Termination Date:	November 14, 2002
Proposed Completion Date:	December 16, 2002

9.0 TEST SUBSTANCE IDENTIFICATION

Two lots of the test substance identified as Kimberly Clark Corporation EPA Registration Number 9402-9 will be used for testing: Code 7345-90A (Saturation date 10-8-02) and Code 7345-91A (Saturation date 10-8-02). The lots of test substance were transferred from Hill Top Research Study Number 02-120919-106 and will be assigned Hill Top Research codes for the generation of the test data.

10.0 TEST SUBSTANCE CHARACTERIZATION

The sponsor will assume responsibility for test substance characterization according to 40 CFR Part 160.105.

11.0 TEST SYSTEM JUSTIFICATION

The test system is designated by federal regulations: EPA Interim Guidance "Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated Towelettes (April 12, 2001)."

12.0 TEST SYSTEM IDENTIFICATION

The test organism to be used in this study will be *Listeria monocytogenes*, ATCC 15313 with 5% Fetal Bovine Serum incorporated as the soil load according to EPA Draft Method Guidance #02, April 12, 2001. These organisms will be assigned a unique code to provide for the correct generation of data.

13.0 TEST PROCEDURE

13.1 The study will be conducted according to the Germicidal and Detergent Sanitizing Action of Disinfectants Method as described in Chapter 6, Disinfectants, Official Methods of Analysis of AOAC International, 17th Edition, 2000 Section 6.3.03 (Appendix I) with modifications and the EPA Interim Guidance "Non-Residual Sanitization of Hard Inanimate Food Contact Surfaces Using Pre-Saturated

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13.0 **TEST PROCEDURE CON'T.**

Towelettes (April 12, 2001)." Records will be maintained to verify compliance with these procedures, and any approved modifications to these procedures.

13.2 The wipes (test substance) will be tested against the test organism containing 5% Fetal Bovine Serum to represent a soil load.

13.3 The wipes will be tested as received from the sponsor. The wipe will be 12" X 12" and removed from the roll immediately prior to testing. **[Two sheets will be removed from the roll and discarded prior to removing the test sheet.]** One (12" X 12") wipe will be used to wipe 4 pans/carriers. One wipe will be used to wipe the specified total test surface area represented by wiping consecutive carriers. The surface area wiped per carrier measures approximately 13.25" X 11" allowing each pan to represent 1 square foot of wiped test surface (4 pans represent 4 square feet of surface area wiped). **[The wipe will be folded two times in half so that each separate folded portion of the wipe will wipe one of each of the four test pans.]**

13.4 Exposure conditions will be for **30 seconds** at 23 ±1°C **after a wiping time of 30 seconds.**

13.5 The neutralizer will be AOAC Neutralizer Blanks with Sea Sand in 400-mL amounts. Neutralizer effectiveness will be determined according to Hill Top Research Standard Operating Procedure 11-DEPP-20-0015A with both test organisms using the plastic pan. The neutralizer will be added to the wiped pan and then the surface will be rubbed [~34 times in the vertical position, ~18 times in the horizontal position, and once around the entire edge (repeat 2 times) in a period of approximately 1 minute] with a sterile rubber policeman to remove the bacteria.

13.6 Other modifications to the AOAC method are as follows:

1) The organisms will be harvested using 1.5 mL of AOAC Phosphate Buffer Dilution Water per bottle instead of 3.0 mL as listed in Section 6.3.03D of the AOAC method.
2) A 0.8-mL aliquot of the adjusted test culture suspension (**~1.5 ±0.5 x 10^8**) will be used to inoculate each gridded test surface (5 and 7/8 " X 8") so that each area of test surface (1 pan/1 square foot) will be inoculated to contain approximately 2.8 X 10^7 CFU/carrier for 1 pan [1 sq. ft.] yielding a count of ~7.5-12.5 X 10^7 CFU/total surface area. **The inoculum will be spread evenly over the pan in a drop-wise fashion so that rows of drops are applied to the rough area on the bottom of each plastic pan (a pattern consisting of ~8-9 drops X ~10-11 drops).** The pans will then be allowed to **air dry for 40 min at 37±2 °C and a relative humidity of at least 50%.** The pans will be loosely covered with aluminum foil during the drying process.

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13.0 **TEST PROCEDURE CON'T.**

3) Numbers controls for each test organism will be conducted using four pans to give the final count per total surface area (4 square feet).
4) Growth will be confirmed by macroscopic examination rather than the method listed in Section 6.3.03J of the AOAC method.
5) The recovery medium will be Brain Heart Infusion (BHI) Agar with 25 mL/L AOAC Stock Neutralizer. Incubation will be at 35 ± 2 °C for 48 ± 2 hours. Plating will be conducted within thirty minutes of neutralizing the test substance by the Pour Plate Method. Two, 10-mL amounts (10^{-1}) of the AOAC Neutralizer Blanks with Sea Sand will be plated across three plates and duplicate 1-mL and 0.1-mL amounts ($10^{-2}$and 10^{-3} dilutions) will be pour plated. [AOAC Phosphate Buffer Dilution Water with Sea Sand (400-mL) and AOAC Phosphate Buffer Dilution Water (9-mL) and BHI Agar will be used for the numbers controls.] **Plate counts will be conducted in duplicate (a + b) and averaged for each pan. Colony counts per milliliter will be multiplied by 4 to yield Colony forming Units (CFU's) per square foot.**

13.7 Observations of conditions during the test will be recorded in the study records.

13.8 Plate counts will be conducted on the expressed fluid from the wipes **immediately** after wiping the pans (carriers). Dilutions will be conducted in 9.9 mL or 9 mL volumes of AOAC Neutralizer Blanks with plating as outlined in Section 13.6(4 with modifications to account for use of 100 mL of diluent in place of 400 mL of diluent.

13.9 The percent reduction in numbers of test bacteria per square foot of surface area (each pan) will be determined as follows:

For each pan

$$\%\ \text{Reduction/ 1sq. ft.} = \frac{\left[\text{Mean (Avg.) of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{8}\right] - \left[\text{Survivors of Individual Pan } \frac{(a+b)}{2}\right]}{\left[\text{Mean (Avg.) of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{8}\right]} \times 100$$

13.10 The percent reduction in numbers of test bacteria per total surface area will be determined as follows:

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13.0 **TEST PROCEDURE CON'T.**

For each of the 4 pan set (4 square feet)
% Reduction/ 4 square feet. =

$$\frac{\left[\text{Sum of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{2}\right] - \left[\text{Sum of Survivors } \frac{(\text{Pan 1a} + \ldots 4\text{b})}{2}\right]}{\left[\text{Sum of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{2}\right]} \times 100$$

13.11 The percent reduction in numbers of test bacteria surviving in the expressed fluid from the wipe will be determined as follows:

$$\frac{\left[\text{Sum of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{2}\right] - [\text{Survivors from Expressed Fluid}]}{\left[\text{Sum of Numbers Control } \frac{(\text{Pan 1a} + \ldots \text{Pan 4b})}{2}\right]} \times 100$$

14.0 **STATISTICAL METHOD**

No statistical analysis is required to interpret the results of this study.

15.0 **REPORT**

A draft report will be issued, for review by the sponsor, prior to issuing the final report. The report will include (but not be limited to) identification of the test organism, test procedure, protocol modification (if any), identification of the test material, solvent (if any), test concentration, subculture media, results, and summary.

16.0 **DATA RETENTION**

The final report and a copy of the raw data will be sent to the sponsor following completion of the study. All records that would be required to reconstruct the study and demonstrate adherence to the Protocol will be maintained. Following completion of the study, the original raw data and the original of the final report will be maintained indefinitely in the form of hard copy to comply with EPA record keeping regulations. The testing facility will retain a copy of these study records in the form of microfilm.

Upon completion of testing, the test substance will be held for one month and then destroyed; or, at your request and cost, sent back to you.

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17.0 NOTICE

If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study.

Similarly, the sponsor will be notified as soon as is practical whenever an event occurs that is unexpected and may have an effect on the validity of the study.

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PROTOCOL APPROVAL FORM
MICROBIOLOGICAL SERVICES DIVISION
HILL TOP RESEARCH, INC.

PROTOCOL TITLE	REFERENCE CODE
Modified AOAC Germicidal and Detergent Sanitizing Action of Disinfectants-Rough Plastic	DISP\PRO\GERM.SAN\KIMC

PROTOCOL APPROVED FOR: HILL TOP RESEARCH, INC.

BY: Kathleen A. Baxter 11-11-02
Kathleen A. Baxter, B.S. Date
Study Director
Microbiological Services Division

Protocol Approved By (Sponsor's Representative):

Rhonda D. J 11-11-02
Signed: Rhonda D. Jones Date
Title: Agent for Kimberly-Clark Corporation
Scientific & Regulatory Consultants, Inc.
PO Box 1014
Columbia City, IN 46725

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APPENDIX I

AOAC Method

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6.3.03

AOAC Official Method 960.09
Germicidal and Detergent Sanitizing Action of Disinfectants

First Action 1960
Final Action

(Suitable for determining minimum concentration of chemical that can be permitted for use in sanitizing precleaned, nonporous food contact surfaces. Minimum recommended starting concentration is 2–4× this concentration. Test also determines maximum water hardness for claimed concentrations. As control, check accuracy of hard-water tolerance results with pure C_{14} alkyl dimethyl benzyl ammonium chloride at 700 and 900 ppm (μg/mL) hardness, and pure C_{16} alkyl dimethyl benzyl ammonium chloride [Cetalkonium Chloride], at 400 and 550 (μg/mL) ppm hardness, expressed as $CaCO_3$.)

A. Reagents

(a) *Culture media.*—(*1*) *Nutrient agar A.*—Boil 3 g beef extract, 5 g peptone (from Difco No. 0118 or equivalent; special grades must not be used), and 15 g salt-free agar in 1 L H_2O. Do not use premixed, dehydrated media. Tube, and autoclave 20 min at 121°C. Use for daily transfer of test culture. (*2*) *Nutrient agar B.*—Prepare as above but use 30 g agar. Use for growing test cultures in French square bottles. (*3*) *Nutrient agar (AOAC).*—*See* 955.11A(c) (*see* 6.1.01). Use for preparing stock culture slants.

(b) *Subculture media.*—(*1*) Use tryptone glucose extract agar (Difco No. 0002), adding 25 mL stock neutralizer, (c),/L. (*2*) Tryptone glucose extract agar (Difco).

(c) *Neutralizer stock solution.*—Mix 40 g Lecithin (Alcolec Granules, American Lecithin, PO Box 1908, Danbury, CT 06813, USA [25–50 kg containers only] or Advanced Lecithin Products, PO Box 677, Danbury, CT 06804, USA), 280 mL polysorbate 80, and 1.25 mL phosphate buffer, (e); dilute with H_2O to 1 L and adjust to pH 7.2. Dispense in 100 mL portions and autoclave 20 min at 121°C.

(d) *Neutralizer blanks.*—For use with ≤200 ppm quaternary ammonium compound. Mix 100 mL neutralizer stock solution, (c), 25 mL 0.25M phosphate buffer stock solution, (e), and 1675 mL H_2O. Dispense 9 mL portions into 20 × 150 mm tubes. Autoclave 20 min at 121°C.

(e) *Phosphate buffer stock solution.*—0.25M. Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1M NaOH, and dilute to 1 L.

(f) *Phosphate buffer dilution water.*—Add 1.25 mL 0.25M phosphate buffer stock solution, (e), to 1 L H_2O and dispense in 99 mL portions. Autoclave 20 min at 121°C.

(g) *Test organisms.*—Use *Escherichia coli* ATCC No. 11229 or *Staphylococcus aureus* ATCC 6538. Incubate 24 and 48 h, respectively. Maintain stock cultures on nutrient agar (AOAC), (a)(*3*), at refrigerator temperature.

B. Resistance to Phenol of Test Cultures

Determine resistance to phenol at least every 3 months by 955.11 (*see* 6.1.01). Resistance of *E. coli* should be equivalent to that specified for *S. typhi* in 955.11D (*see* 6.1.01) and that for *S. aureus* equivalent to that specified for this organism in 955.12 (*see* 6.1.02); also, use procedures under 991.48A(b) (*see* 6.2.03) for *S. aureus*.

C. Apparatus

(a) *Glassware.*—250 mL wide-mouth Erlenmeyers; 100 mL graduate; Mohr, serological, and/or bacteriological (APHA specification) pipets; 20 × 150 mm test tubes. Sterilize at 180°C in hot air oven ≥2 h.

(b) *Petri dishes.*—Sterile.

(c) *French square bottles.*—175 mL, flint glass.

(d) *Water bath.*—Controlled at 25°C.

D. Preparation of Culture Suspension

From stock culture inoculate tube of nutrient agar *A*, A(a)(*1*), and make ≥3 consecutive daily transfers (≤30), incubating transfers 20–24 h at 35–37°C. Do not use transfers >30 days. If only 1 daily transfer has been missed, no special procedures are required; if 2 daily transfers are missed, repeat with 3 daily transfers.

Prepare 175 mL French square culture bottles containing 20 mL nutrient agar *B*, A(a)(*2*), autoclave 20 min at 121°C, and let solidify with bottle in horizontal position. Inoculate culture bottles by washing growth from slant with 5 mL phosphate buffer dilution H_2O, A(f), into 99 mL phosphate buffer dilution H_2O, and adding 2 mL of this suspension to each culture bottle, tilting back and forth to distribute suspension; then drain excess liquid. Incubate 18–24 h at 35–37°C, agar side down. Remove culture from agar surface of 4 or more bottles, using 3 mL phosphate buffer dilution H_2O and glass beads in each bottle to suspend growth. Filter suspension through Whatman No. 2 paper prewet with 1 mL sterile phosphate buffer, and collect in sterile tube. (To hasten filtration, rub paper gently with sterile policeman.) Standardize suspension to give average of 10×10^8 organisms/mL by dilution with sterile phosphate buffer dilution H_2O, A(f).

Table 960.09A Percent light transmission at various wavelengths corresponding to bacterial concentrations

% Light transmission with filters, nm							Average bacterial count/mL
370	420	490	530	550	580	650	
7.0	4.0	6.0	6.0	6.0	7.0	8.0	13.0×10^8
8.0	5.0	7.0	7.0	7.0	8.0	9.0	11.5
9.0	6.0	8.0	8.0	8.0	9.0	10.0	10.2
10.0	7.0	9.0	9.0	9.0	11.0	11.0	8.6
11.0	8.0	10.0	10.0	10.0	12.0	13.0	7.7
13.0	9.0	12.0	12.0	12.0	13.0	15.0	6.7

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Table 960.09B Preparation of $BaSO_4$ suspensions corresponding to bacterial concentrations

Standard No.	2% $BaCl_2$ solution, mL	1% H_2SO_4 (v/v) solution, mL	Average bacterial count/mL
1	4.0	96.0	5.0×10^8
2	5.0	95.0	7.5
3	6.0	94.0	8.5
4	7.0	93.0	10.0
5	8.0	92.0	12.0
6	10.0	90.0	13.5
7	12.0	88.0	15.0

If Lumetron colorimeter is used, dilute suspension in sterile Lumetron tube to give % *T* according to Table 960.09A.

If McFarland nephelometer and $BaSO_4$ standards are used, select 7 tubes of same id as that containing test culture suspension. Place 10 mL of each suspension of $BaSO_4$, prepared as indicated in Table 960.09B, in each tube and seal tube. Standardize suspension to correspond to No. 4 standard.

E. Synthetic Hard Water

Prepare *Solution 1* by dissolving 31.74 g $MgCl_2$ (or equivalent of hydrates) and 73.99 g $CaCl_2$ in boiled distilled H_2O and diluting to 1 L. Prepare *Solution 2* by dissolving 56.03 g $NaHCO_3$ in boiled distilled H_2O and diluting to 1 L. *Solution 1* may be heat sterilized; *Solution 2* must be sterilized by filtration. Place required amount *Solution 1* in sterile 1 L flask and add ≥600 mL sterile distilled H_2O; then add 4 mL *Solution 2* and dilute to 1 L with sterile distilled H_2O. Each mL *Solution 1* will give a water equivalent to ca 100 ppm of hardness calculated as $CaCO_3$ by formula:

$$\text{Total hardness as ppm } (\mu g/mL)\ CaCO_3 = 2.495 \times \text{ppm } (\mu g/mL)\ Ca + 4.115 \times \text{ppm } (\mu g/mL)\ Mg$$

pH of all test waters ≤2000 ppm (μg/mL) hardness should be 7.6–8.0. Check prepared synthetic waters chemically for hardness at time of tests, using following method or other methods described in APHA, *Standard Methods for the Examination of Water and Wastewater* 20th Ed., 1998.

F. Hardness Method

(a) *EDTA standard solution.*—Dissolve 4.0 g $Na_2H_2EDTA{\cdot}2H_2O$ and 0.10 g $MgCl_2{\cdot}6H_2O$ in 800 mL H_2O and adjust by subsequent dilution so that 1 mL of solution is equivalent to 1 mg $CaCO_3$ when titrated as in (c). Check EDTA solution after preparation or, if commercially purchased, against $CaCO_3$ standard at least every 2 months.

(b) *Calcium standard solution.*—1 mL = 1 mg $CaCO_3$. Weigh 1.00 g $CaCO_3$, dried overnight or longer at 105°C, into 500 mL Erlenmeyer and add dilute HCl through funnel until $CaCO_3$ is dissolved. Add 200 mL H_2O, boil to expel CO_2, and cool. Add few drops methyl red indicator and adjust color to intermediate orange with dilute NH_4OH or HCl as required. Transfer quantitatively to 1 L volumetric flask and dilute to volume.

(c) *Determination.*—Dilute 5–25 mL test sample (depending on hardness) to 50 mL with H_2O in Erlenmeyer or casserole. Add 1 mL buffer solution (67.5 g NH_4Cl and 570 mL NH_4OH diluted to 1 L with H_2O), 1 mL inhibitor (5.0 g $Na_2S{\cdot}9H_2O$ or 3.7 g $Na_2S{\cdot}5H_2O$ dissolved in 100 mL H_2O), and one or 2 drops indicator solution (0.5 g Chrome Black T in 100 mL 60–80% alcohol). Titrate with EDTA standard solution slowly, stirring continuously, until last reddish tinge disappears from solution, adding last few drops at 3–5 s intervals.

$$\text{Hardness as mg } CaCO_3/L = (\text{mL standard solution} \times 1000)/\text{mL test sample}$$

G. Preparation of Test Samples

Use composition declared or determined as guide to test sample weight required for volume sterile H_2O used to prepare 20 000 ppm (μg/mL) solution. From this stock dilution, transfer 1 mL into 99 mL of the water to be used in test to give concentration of 200 ppm (μg/mL). In making transfer, fill 1 mL pipet and drain back into stock solution; then refill, to correct for adsorption on glass. After mixing, discard 1 mL to provide 99 mL of the test water in H.

H. Operating Technique

Measure 99 mL water to be used in test, containing bactericide at concentration to be tested, into chemically clean, sterile, 250 mL wide-mouth Erlenmeyer and place in constant temperature bath until it reaches 25°C, or ≥20 min. Prepare duplicate flasks for each germicide to be tested. Also prepare similar flask containing 99 mL sterile phosphate buffer dilution H_2O, A(f), as "initial numbers" control.

Add 1 mL culture suspension to each test flask as follows: Whirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at point of contact with test water. Add suspension midway between center and edge of surface with tip of pipet slightly immersed in test solution. Avoid touching pipet to neck or side of flask during addition. Transfer 1 mL portions of this exposed culture to neutralizer blanks exactly 30 and 60 s after addition of suspension. Mix well immediately after transfer.

For "numbers control" transfer, add 1 mL culture suspension to 99 mL sterile phosphate dilution H_2O in same manner. In case of numbers control, plants need be made only immediately after adding and mixing thoroughly ≤30 s. (It is advantageous to use milk pipets to add culture and withdraw test samples.)

Plate from neutralizer tube to agar, using subculture medium A(b)(*1*) for quaternary ammonium compounds and A(b)(2) with numbers control. Where 0.1 mL portions are planted, use 1 mL pipet graduated in 0.1 mL intervals. For dilutions to give countable plates, use phosphate buffer dilution H_2O, A(f). For numbers control, use following dilution procedure: Transfer 1 mL exposed culture (1 mL culture suspension transferred to 99 mL phosphate buffer dilution H_2O in H_2O bath) to 99 mL phosphate buffer dilution H_2O, A(f), (*dilution 1*). Shake thoroughly and transfer 1 mL *dilution 1* to 99 mL phosphate buffer dilution H_2O, A(f), (*dilution* 2). Shake thoroughly and transfer 1 mL *dilution* 2 to 99 mL phosphate buffer dilution H_2O (*dilution 3*). Shake thoroughly and transfer four 1 mL and four 0.1 mL aliquots from *dilution 3* to individual sterile Petri dishes.

For test samples, use following dilution procedure: Transfer 1 mL exposed culture into 9 mL neutralizer, A(d). Shake and transfer four 1 mL and four 0.1 mL aliquots to individual sterile Petri dishes. For numbers control, use subculture medium A(b)(2); for tests with qua-

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ternary ammonium compounds, use medium A(b)(*1*). Cool agar to solidify, and then invert and incubate 48 h at 35°C before counting.

I. Results

To be considered valid, results must meet standard effectiveness: 99.999% reduction in count of number of organisms within 30 s. Report results according to actual count and percent reduction over numbers control. Counts on numbers control for germicide test mixture should fall between 75 and 125 × 10^6/mL for percent reductions to be considered valid.

J. Sterility Controls

(a) *Neutralizer.*—Plate 1 mL from previously unopened tube.

(b) *Water.*—Plate 1 mL from each type of water used.

(c) *Sterile distilled water.*—Plate 1 mL. After counting plates, confirm that surviving organisms are *E. coli* by transfer to brilliant green bile broth fermentation tubes or lactose broth and EMB agar; confirm *S. aureus* by microscopic examination.

References: *Am. J. Public Health* 38, 1405(1948).
J. Milk Food Technol. 19, 183(1956).
Fed. Regist. 21, 7020(1956).
JAOAC 41, 541(1958); 56, 308(1973).

6.3.04

AOAC Official Method 961.02
Germicidal Spray Products as Disinfectants

First Action 1961
Final Action 1964

(Suitable for determining effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces.)

A. Reagents

Use culture media and reagents specified in 991.47A(a) and (f) (*see* 6.2.02); 991.48A(a) (*see* 6.2.03); and 991.49A(a) and (b) (*see* 6.2.05).

Use as test organisms *Trichophyton mentagrophytes* ATCC No. 9533, prepared as in 955.17D (*see* 6.3.02), to which has been added 0.02 mL octyl-phenoxy-polyethoxy-ethanol (Triton ×100, Union Carbide Corp.)/10 mL suspension to facilitate spreading, *Salmonella choleraesuis* ATCC No. 10708, maintained as in 991.47A(b) (*see* 6.2.02), *Staphylococcus aureus* ATCC No. 6538, maintained as in 991.48A(b) (*see* 6.2.03), and *Pseudomonas aeruginosa* ATCC No. 15442, maintained as in 991.49A(c) (*see* 6.2.05). Incubate all bacterial cultures for 48 h, except *pseudomonas*.

B. Apparatus

Use apparatus specified in 991.47B(a), (b), (e), (n), and (o) (*see* 6.2.02), and in addition:

(a) *Capillary pipets.*—0.1 mL, graduated to deliver 0.01 mL. Sterilize in air oven 2 h at 180°C.

(b) *Microscope slides.*—Noncorrosive, 25 × 25 mm (1 × 1 in.), or 18 × 36 mm glass slide. Sterilize by placing individual slides in Petri dish matted with 2 pieces 9 cm filter paper (Whatman No. 2, or equivalent) in air oven 2 h at 180°C.

(c) *Bacteriological culture tubes.*—Pyrex, 32 × 200 mm (Bellco Glass, Inc., PO Box B, Vineland, NJ 08360, USA).

(d) *Metal forceps.*—Sharp points, straight, 115 mm long.

C. Operating Technique

Thoroughly shake 48 h nutrient broth cultures of *S. choleraesuis* and *S. aureus* and let settle 10 min. For *P. aeruginosa*, follow preparation of culture under 991.49A(c) (*see* 6.2.05). With sterile capillary pipet or sterile 4.0 mm loop, transfer 0.01 mL culture onto 1 sq in. sterile test slide in Petri dish and immediately spread uniformly over entire area. Cover dish immediately and repeat operation until 12 slides have been prepared for each organism. (Use 2 slides as control.) Dry all slides 30–40 min at 37°C.

Spray 10 slides for specified time and distance. If no time or distance specified, use 10 s at 1 ft. (30 cm). Hold each slide 10 min, drain off excess liquid, and transfer slide to individual 32 × 200 mm tube containing 20 mL appropriate subculture medium, 955.11A(d) (*see* 6.1.01), with flamed forceps. Shake culture thoroughly. If broth appears cloudy after 30 min, make subculture to fresh individual tubes of subculture broth. Transfer 2 unsprayed slides as viability controls, to individual subculture tubes in same manner.

Incubate all tubes used for primary and secondary transfers 48 h at 37°C. Read as + (growth) or – (no growth). Killing of test organisms in 10 of 10 trials is presumptive evidence of disinfecting action.

For procedures to be followed in assuring standard cultures, for *S. choleraesuis*, *see* 991.47A(b) (*see* 6.2.02), for *S. aureus*, 991.48A(b) (*see* 6.2.03), for *P. aeruginosa*, *see* 991.49A(c) (*see* 6.2.05). For *T. mentagrophytes*, *see* 955.17A and D (*see* 6.3.02).

If there is reason to believe that lack of growth in subtransfer tubes is due to bacteriostasis, inoculate all incubated subculture tubes with loop needle inoculation of respective test culture and reincubate. Growth of these inocula eliminates bacteriostasis as cause of lack of growth. If there is question as to possibility of contamination as source of growth in subculture tubes, make gram stains and/or subculture for identification, according to respective test culture.

If fungicidal activity as well as germicidal activity is involved, use test suspension of *T. mentagrophytes* spores, 955.17D (*see* 6.3.02), and prepare 12 slides, using 0.01 mL standard spore suspension, spraying and subculturing exactly as above. Make subcultures in glucose broth, 955.17B (*see* 6.3.02), incubating 7 days at 25–30°C.

References: *JAOAC* 44, 422(1961); 50, 763(1967).
Soap Chem. Spec. 38(2), 69(1962); 61, 400(1978).

6.3.05

AOAC Official Method 966.04
Sporicidal Activity of Disinfectants

First Action 1966
Final Action 1967

(Suitable for determining sporicidal activity of liquid and gaseous chemicals. Applicable to germicides for determining presence or absence of sporicidal activity against specified spore-forming bacteria in various situations and potential efficacy as sterilizing agent.)

A. Reagents

(a) *Culture media.*—(*1*) *Soil extract nutrient broth.*—Extract 1 lb (454 g) garden soil in 1 L H_2O, filter several times through S&S No. 588 paper, and dilute to volume (pH should be ≥5.2). Add 5 g